The Neonatal Salivary Transcriptome

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The ability to noninvasively assess the physical and developmental status of a neonate is a goal of modern medicine. In recent years, technological advances have permitted the high-throughput analysis of saliva for thousands of genes, proteins, and metabolites from a single sample source. Saliva is an ideal biofluid to assess health, disease, and development in the newborn. It may be harnessed repeatedly, even in the most vulnerable patients, without risk of harm. Translating novel information about an infant's global development and risk of disease to the neonatal bedside through the salivary transcriptome has the potential to significantly improve clinical care and outcomes in this at-risk population.

Neonates, particularly those born premature, present unique challenges to caregivers. Their small size, clinical fragility, and limited blood volumes often prohibit invasive testing and may limit enrollment into clinical trials aimed at improving care through the development and implementation of novel treatment strategies. Thus, utilizing a noninvasive and easily accessible biofluid that provides caregivers with essential information about the health status and development of a newborn holds great promise for significantly improving patient care and outcomes (Bianchi et al. 2010).

Saliva is a diverse source of genetic material (DNA and RNA), proteins, metabolites, and microorganisms. Through both extracellular and intracellular trafficking mechanisms, biomarkers enter into the oral cavity and may be monitored to inform an investigator about overall health and systemic disease in a patient. Saliva has the benefits over other noninvasively obtained biofluids, such as urine or stool, in that

it may be obtained on demand and repeatedly in a limited time frame. These advantages make it an ideal biofluid for noninvasive clinical assessment across a wide variety of healthcare settings, including in the vulnerable newborn population (Wong 2012; Schafer et al. 2014).

Although salivary studies identifying single proteins (e.g., cortisol) (Maganano et al. 1992) or microorganisms (e.g., cytomegalovirus) (Balcarek et al. 1993) in the newborn were performed more than 20 years ago, genomic, transcriptomic, and proteomic salivary analyses are relatively new fields of discovery. Arguably, the vast majority of preterm infants are born without genetic syndromes. Rather, their unique phenotypes, diseases, and developmental impairments are a consequence of the effects of preterm birth on transcriptional and translational processes and epigenetic modifications. Gaining access, in real-time, to ongoing disrupted developmental patterns at a molecular level, particularly at the level of the transcriptome,

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may allow caregivers an unprecedented opportunity to identify infants at risk for specific neonatal systemic and/or developmental sequelae. Utilizing saliva to further our understanding of complex transcriptional regulatory mechanisms may also identify novel targets for intervention and therapy for those born premature.

This review will highlight the recent emergence of the salivary transcriptome as a novel and noninvasive means to access real-time genetic information to assess clinical status and disease susceptibility. Specific emphasis will be placed on the diagnostic and potentially prognostic potential of salivary transcriptomics in the premature newborn population and focus on the enormous amount of developmental information available to caregivers from a single sample source. It will address unique challenges associated with salivary analysis in the newborn and review alternative strategies to ensure that these techniques translate to neonatal care. Finally, it will highlight specific applications of salivary transcriptomics in the premature newborn in which ongoing assessment of developmental skills may not only improve short- and long-term patient care and outcomes but also reduce health care costs.

SALIVA AS A BIOFLUID

Despite its reputation as a rich source of biomarkers, the vast majority of saliva is water. Only 1% of the volume contains a mixture of electrolytes, microorganisms, proteins, and genetic material. Ninety percent of saliva is formed in the major salivary glands (parotid, submandibular, and sublingual), with 10% forming in the minor glands (labial, buccal, and palatal) (Cuevas-Córdoba and Santiago-García 2014). As a direct filtrate of blood, saliva shares many of the same biomarkers found in plasma. Studies in adults comparing the proteomic profiles of plasma and serum from the same individuals revealed a 27% similarity between biofluids (Loo et al. 2010). Similarly, comparative transcriptomic analyses between umbilical cord blood and neonatal saliva revealed ~40% similarity in genes expressed at the highest quintile in each biofluid (Maron et al. 2012a). Saliva is

also a rich source of microorganisms, including viruses, bacteria, and fungi, whose composition may serve as a window into the overall health status of an individual. Comprehensive reviews of adult saliva have been previously written and can be found elsewhere (Lawrence 2002; Llena-Puy 2006; Tiwari 2011; Cuevas-Córdoba and Santiago-García 2014).

The saliva of a newborn differs from its adult counterpart because of several distinct characteristics. First, newborns lack teeth. Because a large proportion of microorganisms in the oral cavity are located near teeth, the microbiotic component of a newborn's saliva differs from adults and older children (Pennisi 2005). Second, colonization with microorganisms in the oropharynx occurs rapidly in the days to weeks following birth. Premature newborns are often delivered via cesarean section, may not feed for days, and have instrumentation with nonsterile nasogastric and endotracheal tubes that may lead to additional disruption of microbiotic colonization. Finally, there have been few, if any, studies conducted that examine the impact that the developing salivary glands may have on salivary constituents in the human newborn. It is possible that filtration and diffusion mechanisms are affected by the ongoing development of each gland, further altering salivary substrates in a neonate. Although these effects may not influence data obtained on neonatal subjects, care should be made when extrapolating adult saliva studies to this population and/or combining samples obtained from different developmental stages.

SALIVARY COLLECTION, STORAGE AND PROCESSING

Historically, RNA's single stranded structure, along with the oropharynx's ubiquitous RNAses, had made RNA highly susceptible to rapid degradation. However, for over a decade, commercially available salivary collection kits have made it possible for investigators to interrogate both messenger RNA (mRNA) and regulatory or microRNA (miRNA) in saliva samples. An essential first step to salivary transcriptomics is the rapid stabilization of the sample by inhibit-

ing RNases, halting gene expression changes and limiting microbial overgrowth within minutes of salivary collection. Comparative studies analyzing the effectiveness of varying salivary stabilizing solutions for downstream transcriptomic analysis have been performed. Wong (2007) reported that RNeasy is a poor stabilizing reagent because of its high salt content and ability to increase cellular membrane permeability, which increases the release of contaminating genomic DNA. In 2011, Lee et al. (2011) described a direct saliva transcriptome analysis that successfully used an ambient-temperature processing, stabilization and storage protocol for salivary RNA. More recently, Pandit and colleagues (2013) have modified an existing Qiagen protocol (QIAzol), to provide investigators with a robust, cost-effective alternative to other commercially available RNA extraction kits. By comparison, published reports in the newborn have successfully used Qiagen's RNeasy Protect Saliva Solution on multiple downstream transcriptomic platforms (Maron et al. 2010, 2012b; Dietz et al. 2012). Thus, investigators have options when considering salivary collection and extraction kits and should consider study design, location and costs when choosing a product or protocol.

Collection of saliva samples in the newborn poses additional challenges to the investigator. In older children and adults, subjects can provide passive drool or use appropriate sponges or collection devices for saliva collection. Further, subjects can rinse their mouths out before sample collection to limit food or debris contami-

nation. However, in the newborn, samples must be obtained by the investigator. Thus, there is a theoretical risk of human breast milk contamination in neonatal saliva samples that may alter their gene expression profiles. Dietz and colleagues (2012) have developed an easy to use system that relies on readily available equipment found in most neonatal intensive care units (NICU) and special care nursery settings. Briefly, saliva is collected with a 1 mL syringe, end caps removed, attached to low-wall suction. Saliva is collected in the syringe for 20–30 sec and immediately stabilized at the bedside. Samples may then be left at room temperature, 4°C or frozen at –20°C or –80°C pending processing and manufacturer's guidelines (Dietz et al. 2012). Most commercially available collection kits allow for samples to sit at room temperature for days to weeks without any risk of RNA degradation. Sponges are also available for saliva collection in the newborn, but investigators must take the additional step of centrifugation to retrieve the saliva sample. It should be noted that in the neonatal population, modifications to existing protocols might need to be made to account for the limited salivary volumes available (Table 1) (Maron 2011; Dietz et al. 2012). Most salivary collection kits recommend ≥ 1 mL of saliva for downstream RNA extraction and analysis. However, neonates on average can only generate ≤ 50 μ L of saliva per sample (Maron et al. 2010, 2012b). Despite these small volumes, successful extraction and identification of RNA targets has been performed repeatedly in the newborn. Thus, investigators

Table 1. Unique considerations for salivary analysis in the newborn

	Adults	Neonates
Salivary volume yield	Average collection: 1.5–2 mL	Average collection: 10–50 μ L
Collection devices	Voluntary passive drool Sponges	Sponges Suction catheters Filter paper
Contamination considerations	Limit food and/or drink before salivary collection Rinse mouth before salivary collection	Potential for human breast milk contamination
Processing	Multiple available processing protocols available (Wong 2007; Lee et al. 2011; Pandit et al. 2013)	Modifications may be required because of smaller starting volumes (Dietz et al. 2012)

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should not be deterred by the limited starting saliva volumes or the required protocol modifications when designing studies for the newborn (Table 1).

SALIVARY RNA AND DOWNSTREAM APPLICATIONS

With the availability of RNA stabilizing reagents, the first reports of the detection, identification and diagnostic application of salivary RNAs began emerging. In their original article, Li et al. (2004a) described the identification of salivary mRNAs in healthy adult subjects. The investigators performed whole transcriptomic microarray analyses on saliva samples collected from 10 subjects (six males) and identified what they coined as the Normal Core Salivary Transcriptome (NCST) (Li et al. 2004a). NCST is composed of 185 genes expressed in all study subjects. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) studies confirmed the universal expression of each gene in adult saliva samples. Similarly, Maron et al. (2012a) reported on the neonatal saliva transcriptome and described genes expressed at the highest quintile on gene expression microarrays in all term neonatal study subjects ($n = 15$). Comparative analysis between both published adult and neonatal gene lists by Ingenuity Pathway Analysis (IPA version 21901358; release date December 9, 2014) to examine biological differences in salivary constituents revealed that neonatal saliva had unique gene transcripts related to embryonic development, hair and skin development and function, and organ development not found in adult subjects ($p < 0.01$) (J Maron, unpubl.). These findings further support the unique properties of neonatal saliva.

In 2006, Kumar and colleagues (2006) refuted the notion that salivary RNAs were detectable in human saliva, stating that signals on microarrays and RT-qPCR amplification were caused by genomic DNA contamination. However, a series of published reports have confirmed the presence of saliva mRNAs, leaving little doubt of their potential for enhancing and informing clinical care (Li et al. 2004a; Hu et al. 2006, 2008; Park et al. 2007; Maron et al. 2010; Lee

et al. 2011). Extracted mRNAs have been applied to multiple downstream transcriptomic platforms including microarrays (Li et al. 2004a), RT-qPCR (Maron et al. 2012b), and RNA sequencing (Spielmann et al. 2012). Each technique offers the investigator a slightly different approach to interrogate the transcriptome. Although microarrays and RNA sequencing (RNA-seq) provide the opportunity to interrogate global gene expression, RT-qPCR can provide high-throughput analysis of targeted gene biomarkers. RNAseq has the added benefit of improving our understanding of transcriptional regulatory processes and alternative splicing, particularly in the setting of disease and disrupted development in the newborn. It should be noted, however, that despite available stabilizing agents designed to inhibit bacterial overgrowth, saliva is a mixed species flora that may interfere with sequencing readouts (Spielmann et al. 2012).

Applying knowledge about the salivary transcriptome to disease and development is the ultimate goal of research. In recent years, biomarkers associated with diverse medical conditions have been described. Importantly, these initial biomarker panels extend beyond oral cancer (Li et al. 2004b; Brinkmann and Wong 2011) and autoimmune diseases that affect the oral cavity (e.g., Sjögren syndrome) (Miceli-Richard and Criswell 2014). Rather, emerging reports highlight the diagnostic potential of salivary transcriptomic analysis for identifying ovarian cancer (Lee et al. 2012), respectable pancreatic cancer (Zhang et al. 2010a), lung cancer (Zhang et al. 2012) breast cancer (Zhang et al. 2010b), and sleep deprivation (Seugnet et al. 2006), among others. Each of these studies highlights the potential for noninvasive salivary transcriptomic diagnostics to better identify systemic pathophysiology and disease in adult populations and laid the foundation for the integration and application of salivary transcriptomics into neonatal care.

TRANSLATING THE TRANSCRIPTOME; NEONATAL APPLICATIONS

In 2010, the first published report on neonatal salivary gene expression described the enor-

mous amount of developmental information available in as little as 10 μ L of neonatal saliva (Maron et al. 2010). By analyzing saliva samples obtained serially from five premature infants from the time of birth to discharge, investigators were able to show that global developmental systems could be monitored in real-time. Following comparative whole-transcriptomic microarray analyses aimed at identifying genes that statistically significantly changed their expression pattern over time, the investigators reported that infants down-regulated genes associated with embryonic, hematologic, and connective tissue development, while simultaneously up-regulating genes involved in organ and tissue development, as well as neurodevelopment and behavior as they matured (Maron et al. 2010). This discovery phase study was the first to use neonatal saliva to understand normal and potentially pathologic development at a molecular level. It ultimately generated important hypotheses about oral feeding readiness in premature neonates.

The determination of oral feeding maturity in the preterm infant population remains a clinical challenge because of neurodevelopmental complexity (Lau et al. 2000; Barlow 2009) and its dependence on subjective nursing assessment (Palmer et al. 1993). Current standards of care mandate the use of cue-based feeding protocols that ultimately lack the necessary specificity and sensitivity required to avoid placing infants at risk for choking, desaturation, and feeding aversion. Prior research has shown that for a newborn to successfully feed they must integrate numerous cranial nerves and muscles, along with their sensory, neurodevelopmental, and hunger signaling pathways (Barlow 2009; Maron et al. 2015). Interestingly, in the original neonatal salivary transcriptome study, computational and systems biology reviews of gene targets highlighted biological pathways associated with feeding maturity. Because each study subject learned to orally feed during his/her hospitalization, genes identified in the initial study mapped to biological processes involved in cranial nerve development, facial morphogenesis, neurodevelopment, and feeding behav-

ior (Maron 2011). Within the feeding behavior category, salivary gene expression of the neuropeptide Y2 receptor (*NPY2R*) was subsequently shown to be a highly informative biomarker of feeding immaturity in this population (Maron et al. 2012b).

NPY2R is a well-described hypothalamic gene involved in hunger regulation (Naveilhan et al. 1999; Huang et al. 2008; Hunt et al. 2011). It is one of five known receptors for neuropeptide Y (NPY). Unlike the other NPY receptors, *NPY2R* is the only gene in which expression must be down-regulated to increase hunger (Pjetri et al. 2012). In 2012, Maron and colleagues (2012b) showed that expression of *NPY2R* in neonatal saliva had a 95% positive predictive value for neonatal feeding immaturity. However, despite its strong positive predictive value, its negative predictive value was only 27%. Thus, *NPY2R* represents just one of the biological processes required for successful oral feeding in the newborn.

To address this lack in knowledge, additional neonatal salivary gene expression microarray analyses were conducted on premature newborns during the learning process of oral feeding. Computational analysis and systems biology review of the microarray data identified a panel of genes ($n = 24$) that were involved in the diverse biological systems required in oral feeding maturation including sensory integration, facial development, neurodevelopment, hunger signaling, and digestive system development (Maron et al. 2015). A large prospective validation phase testing each of these biomarkers, alone and in combination, on 400 saliva samples resulted in the further identification of five genes (*NPY2R*, *WNT3*, *PLXNA1*, *NPHP4*, and *AMPK*), that when combined with postconceptional age and sex had good accuracy at predicting feeding success in the newborn (area under the receiving operator curve = 0.78). This research not only laid the foundation for the integration of salivary diagnostics into neonatal care, but clearly showed the utility of exploring the salivary transcriptome to further our understanding of evolving and potentially disrupted development in neonates (Fig. 1).

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Figure 1. Neonatal salivary transcriptomics: Applications. (Modified from OpenClips image on Pixabay © 2015 [http://pixabay.com] under Creative Commons Deed CC0.)

FUTURE DIRECTIONS: RNA SEQUENCING, SALIVARY microRNAs, EXOSOMES AND BEYOND

Detection of mRNAs is only one component of salivary transcriptomics. RNA sequencing and exosome studies have also successfully been performed on adult human saliva samples (Palanisamy et al. 2010; Spielmann et al. 2012; Gallo and Alevizos 2013; Ogawa et al. 2013; Xie et al. 2013; Yang et al. 2014). Both RNA and exosome sequencing studies allow for simultaneous gene expression and transcriptomal regulatory mechanisms. Exosomes are small membrane vesicles that are known to contain miRNAs, proteins, mRNAs, noncoding RNAs (ncRNA), lipids, proteins, and cellular constituents (Thery et al. 2009). Emerging studies have shown that exosomes are essential for cell-to-cell communication throughout the body and play an important role in dissemination of disease (Alečković and Kang 2015), host-pathogen interactions (Schorey et al. 2014), and regenerative medicine (De Jong et al. 2014). Yang and colleagues (2014)

have recently described the detection of tumor cell-specific mRNA in salivary exosome-like microvesicles of a novel human lung cancer xenograft mouse model. Although similar studies in the newborn have yet to be performed, these technologies and approaches nevertheless hold the promise of furthering our understanding of aberrant development in this population.

CONCLUSIONS

Exploration of the salivary transcriptome is poised to enhance the ability to noninvasively monitor the developing, vulnerable newborn. Only simple modifications to existing protocols, combined with creative ways of collecting saliva are needed to gain access to real-time global and systemic developmental information that was never previously possible in the newborn. Technological advances are providing investigators with opportunities to not only interrogate whole exomes, but to further our understanding of transcriptional regulatory processes through RNA sequencing. These advances may ultimate-

ly lead to novel treatment strategies. The detection of circulating exosomes containing regulatory miRNAs may also elucidate cell-to-cell trafficking and aid in diagnostics. Now is the time to integrate this technology into neonatology so that the most vulnerable of patients may benefit from noninvasive salivary diagnostics.

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